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**KOSTOV G.A. PhD head assistant, ANGELOV M.P. associated proffesor PhD ,**
**DENKOVA Z.R. doctor of science proffesor, KOPRINKOVA-HRISTOVA P. associated proffesor PhD,**
**PANDJAROV P. student**

# *CHARACTERISTICS OF YEAST STRAINS FOR ETHANOL FERMENTATION FOR THE PURPOSE OF BIOFUELS AND FOOD INDUSTRIES*

**Abstract.** In this work were studied the fermentation characteristics of yeast strains supposed for ethanol production for the food industry and biofuels. The strains were tested in five different nutrient media containing glucose and sucrose as substrate. Based on the data for the fermentation process' dynamics were determined and the ethanoconcentration, ethanol yield and maximum specific growth rate of the yeast strains. It was selected couples strain-medium and was commented the capabilities to obtain ethanol with these strains.

**Key words:** ethanol fermentation, yeast, biofuels, food inductry, specific growth rate.

Изучены условия спиртового брожения, которые рекомендованы для производства спирта в пищевой промышленности и биотоплива (биомассы). Штаммы дрожжей были проверены в пяти различных средах, содержащих глюкозу и сахарозу, как субстрат (основа). Основываясь на данных динамики брожения были определены прирост концентрации спирта, максимальная скорость прироста дрожжевой биомассы. Отобраны соответствующие пары штамм-среды, которые были рекомендованы для производства.

**Ключевые слова**: спиртовое брожение, дрожжи, биотопливо, дрожжевая биомасса, пищевая промышленность, темп прироста.

1. Intorduction

During last years the ethanol became a possible substituent of the fossil fuels. It is clear, colorless, flammable, oxygenated hydrocarbon with the chemical formula $C_2H_5OH$. Ethanol can be used as a transport fuel in at least four forms: anhydrous ethanol (100% ethanol), hydrous ethanol (95% ethanol and 5% water), anhydrous ethanol-gasoline blends (10–20% ethanol in gasoline) and as raw material for ethyl tert-butyl ether (ETBE). In EU several instruments encouraging bio-fuel and especially ethanol production are approved: The white book "Energy for the future: renewable energy sources" from 1997; The green book "Towards European strategy for energy supplies stability" from 2000; Directive 2003/30/EU for encouragement of bio-fuels and other renewable energy sources utilization in transportation from May 2003 [1,2,3,4,7,10]. Bio-ethanol is obtained by diverse raw materials – wheat, molasses, corn, Jerusalem artichoke, barley, ligno-cellulose materials.

The second major element of ethanol market is alcohol drinks production. Most of them can be classified into two main groups: fermented alcohol drinks and distillated alcohol drinks. The distillated alcohol drinks are produced by distillation of already fermented drinks. In this group is production of pure grain or potato alcohol. Exhaustive information about production of all kinds of alcohol can be found in the literature concerning technology of different drinks production [5]. The third main element of ethanol market is its industrial application. As it was mentioned before, over 150 industries use ethanol as main or accessory raw material. Pure ethanol and 95% purified ethanol are used as dissolvent because of their lower polarity in comparison with water [1].

Ethanol production has several main and mutually related stages: collecting and delivery of raw materials, hydrolysis, fermentation, steam production, distillation and rectification and making harmless of wastes [10].

Ethanol production has two main steps: fermentation and distillation. Fermentation of sugars to ethanol by yeasts has important role in ethanol production. Alcohol fermentation is carried out by highly productive yeast strains from Saccharomyces genera and some bacteria species, mainly Zymomonas mobilis –strain isolated from Mexican alcoholic drinks that ferments glucose twice faster than yeasts. The genera *Saccharomyces* include 41 strains of yeasts. Most of the investigations were carried out with strains of *Saccharomyces cerevisiae* [4, 5, 6]. They ferment the following carbohydrates: glucose, fructose, maltose, sucrose and partially raffinose but are not able to ferment xylose, arabinose and lactose. Some other species of yeasts need carbon sources in the cultural media that are hardly fermentable by S.cerevisiae. Yeasts strains S.uvarum, S.carlsbergensis and S.diastaticus that are able to assimilate dextrin are also used. Yeast strains Kluyveromyces fragilis, Kluyveromyces lactis and some species from genera Candida are able to synthesis enzyme β-galactosidase and can ferment lactose from milk whey [4, 5, 6].

Alcoholic yeasts that produce ethylic alcohol must have the following characteristics: fast reproduction, resistance to high sugar and alcohol concentrations, to be able to transform carbohydrates to alcohol with low waste production of by-products, to be insensitive to environmental changes such as temperature, osmotic pressure, pH etc. [4,6].

The purpose of this work is to make an initial assessment of yeast strains for ethanol fermentation of various fermentation mediums and to select appropriate combinations of strain- medium.

2. Materials and methods

2.1. Strains

The yeast strains *Saccharomyces cerevisiae* (K32, P2, YC, SP, SsoS, C11, C, 46 EVD) with proven ethanol capability fromthe collection of Departments of "Wine and Brewing" and "Organic chemistry and Microbiology" were used in the present work. The strains are stored on malt agar in refrigerator at $4^0$C. Before all experiments the strains was activated growing on malt agar at $28^0$C in thermostat.

2.2. Fermentation mediums

2.2.1. For comparison of ethanol synthesis properties of different yeast strains

A. Inoculums medium (g/dm$^3$): sucrose - 5, $NH_4Cl$ - 0,2, $MgSO_4x7H_2O$ – 0,05, $KH_2PO_4$ – 0,3, $Na_2HPO_4$ – 0,05, yeast extract - 0,15.

B. Fermentation medium (g/dm$^3$): sucrose - 150, $NH_4Cl$ - 4, $MgSO_4x7H_2O$ – 0,5, $KH_2PO_4$ - 2,5, yeast extract - 1.

2.2.2 Fermentation mediums

A. Fermentation medium 1 (FM1) (g/dm$^3$): glucose - 200, $(NH_4)_2SO_4$- 10, $KH_2PO_4$ – 4, yeast extract - 5, peptone – 10, $MgSO_4x7H_2O$ - 1, $CaCl_2$ - 0,2

B. Fermentation medium 2 (FM2) (g/dm$^3$): glucose

– 118,40; $(NH_4)_2SO_4$ – 2; $KH_2PO_4$ – 2,72; $MgSO_4x7H_2O$ – 0,5; yeast extract – 1

C. Fermentation medium 3 (FM3) (g/dm$^3$): sucrose – 220, $NH_4H_2PO_4$ – 1,5, yeast extract – 3, peptone – 1,5, $MgSO_4x7H_2O$ - 3

D. Fermentation medium 4 (FM4) (g/dm$^3$): sucrose – 150, $KH_2PO_4$ – 5, $NH_4Cl$ – 5, yeast extract – 6, $MgSO_4x7H_2O$ – 1, KCl - 1

E. Fermentation medium 5 (FM5) (g/dm$^3$): molasses – 227, $(NH_4)_2SO_4$- 5,2, $KH_2PO_4$ – 1,53, pH – 3,9

All mediums were sterilized for 20 min at 121$^0$C in autoclave.

2.3. Experimental procedures

2.3.1. For comparison of ethanol synthesis properties of different yeast strains

At 8 sterile tubes was spread out on 5 cm$^3$ sterile medium (2.1.1 A). The tubes were inoculated with yeast suspension, obtained by elution with saline of the yeast biomass from the malt agar. Tubes were thermostatted at 28$^0$C per day. With so prepared inoculums the fermentation medium (2.1.1 B) were inoculated. For this purpose 250 cm$^3$ flasks, equipped with fermentation stopper, were filled with 200 cm$^3$ fermentation medium (2.1.1 B) and were inoculated. Flasks were thermostated at 28$^0$C. The accumulation of ethanol was compared to the 5-day from the beginning fermentation.

2.3.2. For comparison of ethanol synthesis properties of different yeast strains in different fermentation mediums

250 cm$^3$ flasks, equipped with fermentation stopper, were filled with 200 cm$^3$ fermentation mediums (2.1.1 C). Each flask was inoculated with 5 ml yeast suspension from the selected yeast strains. The selected strain were seed on malt agar and thermostatted for 24 h at 28$^0$C. The flasks were inoculated with yeast suspension, obtained by elution with saline of the yeast biomass from the malt agar. The results for ethanol concentration are average from 3 independent measurements.

2.3.3. Specific growth rate determination.

The maximal specific growth rate was determined from the fermentation dynamic. At every 12$^{th}$ hour was measured the ethanol concentration and the concentration of substrate in the cultural medium. The ethanol yield is determined by the equation:

$$\alpha = \frac{E}{k.S_0} \qquad (1)$$

where: E – ethanol concentration, g/dm$^3$; к – theoretical yield –(for glucose k=0,51; for sucrose k=0,55; $S_0$ initial substrate concentration, g/dm$^3$;

The specific growth rate was determined by equation:

$$\lambda_P Y = \alpha\mu \qquad (2)$$

where: Y – biomass yield coefficient; For spirit yeast Y=0,035-0,04 [8,9]. In the work Y=0,03.

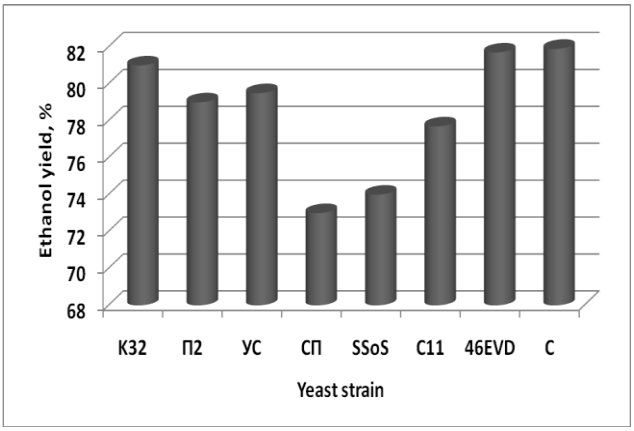
2.4. Analisys

The ethanol and substrate concentration were determined by „Anton Paar DMA 4500", Austria [11].

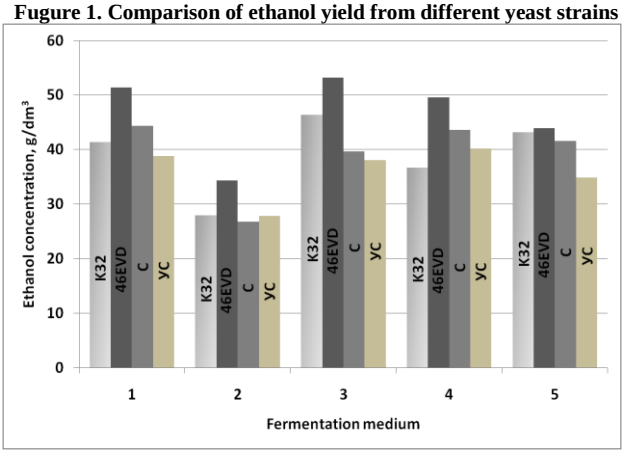
3. Results and discussion

3.1. Comparison of ethanol synthesis properties of different yeast strains.

Experimental work for the selection of strain-producer of ethanol was carried out with 8 yeast strains, that have previously proven good fermentation qualities. Initial comparison of the yeast ability to produce ethanol was made of medium 2.2.1 B. The results of comparing the productivity of alcohol tested strains are shown in Figure 1. Data show that the amount of ethanol produced from different strains was comparable. In this sense, should not be given definite advantage of one of them, moreover, that the final selection can be made after further investigations of their kinetic characteristics and the capability of cultivation in industrial systems.




**Fugure 1. Comparison of ethanol yield from different yeast strains**

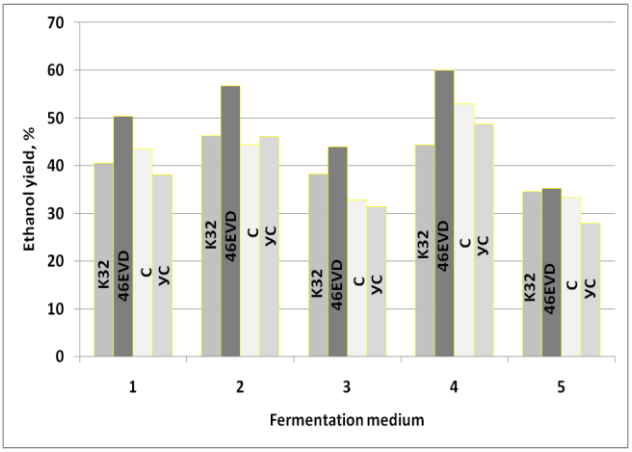



**Fugure 2. Comparison of ethanol concentration from different yeast strains cultivated at different medium**

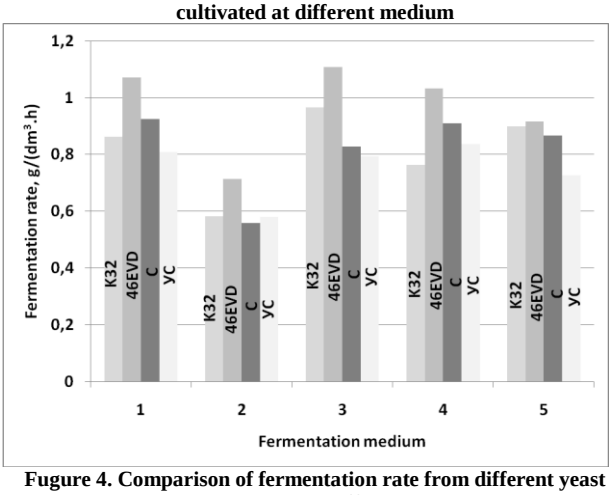
To carry out the following research have been selected the strains *S.cerevisiae* K32, *S.cerevisiae* YC, *S.cerevisiae* 46 EVD and *S.cerevisiae* C. These strains are characterized by the highest yield in primary screening.

3.2 Investigation of selected strains on different nutrient mediums.

The comparison of growth and biosynthetic capabilities of the selected strains on different nutrient media was did at 48 h from the beginning of the fermentation process. As initial parameters for comparison were selected – ethanol concentration in the medium, g/dm$^3$; ethanol yield,%; fermentation rate, g/(dm$^3$.h). Ethanol yield was calculated in accordance with the used substrate. The obtained results are summarized in Figure 2 to Figure 4. On FM 1 a high ethanol concentration was observed for S.cerevisiae 46EVD, followed by S.cerevisiae C. The other two strains had a slightly weaker performance. In accordance with the observed concentration the yield for S.cerevisiae 46 EVD was highest and hence we had the highest average fermentation rate - 1,07 g/(dm$^3$.h). The results obtained in the FM 2 indicate that the highest yield is achieved at 48 h and at a higher average




**Fugure 3. Comparison of ethanol yield from different yeast strains cultivated at different medium**




**Fugure 4. Comparison of fermentation rate from different yeast strains cultivated at different medium**

speed of fermentation was determined for S.cerevisiae 46EVD, followed by S.cerevisiae K32. Unlike the first medium, here the average fermentation rate is lower (0,71 g/(dm$^3$.h)), and the resulting yield is about 60%. Medium with optimized composition and is intended to conduct the fermentation processes with S.cerevisiae 46 EVD with free and immobilized cells. In the immobilized cell fermentation the lower fermentation rate is preferable. It was compensate by the increased amount of cells in the bioreactor working volume as a result of immobilization.

In FM 3, the results again give priority to the K32 and 46EVD. They were characterized again with the highest yield and high specific fermentation rate - within 0,9 - 1,1 g/(dm$^3$.h). Other strains were showing lower potential for fermentation in this medium.

For FM4 the highest fermentation rate had the strains 46EVD, C and YC. For these strains the specific rate of ethanol accumulation was the range 0,83-1,03 g/(dm$^3$.h).

Medium 5 was molasses with a sugar concentration 227 g/dm$^3$. On it, all strains showed approximately equal fermentation rate within 0.8 -1 g/(dm$^3$.h). Molasses provides the necessary sugar and growth factors that determines the high fermentation rate and thus higher ethanol yield. Most strains are cultivated mainly from molasses, which explains the observed rates and fermentation yields of product.

From preliminary studies could be made following initial conclusions for the yeast strains:

1. *S.cerevisiae* K32, *S.cerevisiae* YC, *S.cerevisiae* 46 EVD and *S.cerevisiae* C are characterized with high ability to form ethanol.

2. The best performances of selected nutrient media shows strain *S.cerevisiae* 46 EVD. It shown higher specific rates of product accumulation and consequently the highest ethanol yields of ethanol.

3. The strains *S.cerevisiae* K32 and S.cerevisiae 46EVD are suitable for cultivation in medium containing glucose as a substrate.

4. All tested strains are suitable for the cultivation in nutrient media containing sucrose as a substrate.

5. All strains grow on with a maximum rate on molasses, making them suitable for industrial application.

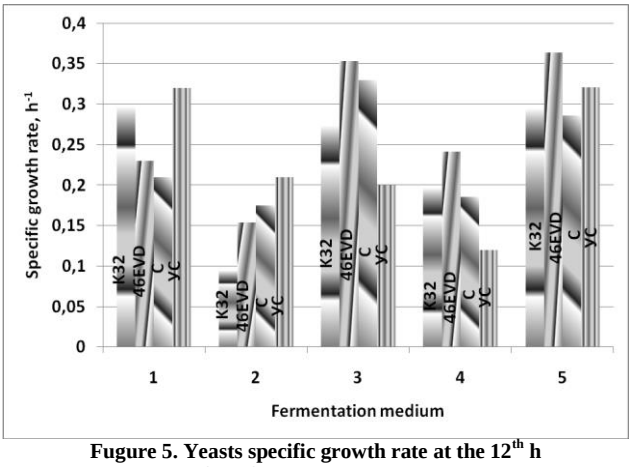
3.3 Investigation the fermentation dynamics and comparison of strains by the specific growth rate.

For more precise selection of pairs strain/growing media should be aware and the kinetic characteristics of the yeasts and especially the specific growth rate of the strain in the culture medium. For this purpose, it was studied the dynamics of accumulation of ethanol in each of the five nutrient media. The dynamics of the fermentation process was monitored 48 th hour, and at every 12 h was determined the ethanol concentration. Based on data from the dynamics of the fermentation process (results are not show in the work) was calculated the specific rate of growth of microorganisms in accordance with dependencies in 2.3.3. In all strains tested the maximal specific growth rate was observed between 12 th and 24 th hour of the beginning of the fermentation process. Graphs of the specific growth rates of tested strains are shown in Figure 5 and Figure 6. The results of cultivation of strain K32 on FM 1 show that the maximum specific growth rate of 0,29 h$^{-1}$ was observed between 12 th and 24 th hour of the start of the process. At 24 th hour, a slight reduction in this parameter due to the depletion of sugars in the medium. At FM 2 the strain developed worst and consequently there was lower specific growth rate. Maximum value of this parameter was observed on 24 th hour - 0,176 h$^{-1}$. In the cultivation of K32 in FM 3 is reached relatively high fermentation rate 1,2-1,3 g/(dm$^3$.h). Rate decreases sharply at a concentration of ethanol in the middle of about 30 g/dm$^3$, which corresponds to a yield of 25% during fermentation of 48 h. This is due to the influence of product inhibition and the depletion of sugars in the medium. The influence of product inhibition in this cultural medium is stronger because of the high initial concentration of sugars and the relatively low yield. On 12$^{th}$ h was observed specific growth rate is 0,274 h$^{-1}$, and 24$^{th}$ h of the process is - 0,352 h$^{-1}$. On FM 4 data are respectively - 12 h - 0,197 h$^{-1}$ and 24 h - 0,24 h$^{-1}$. In the cultivation of molasses seen the highest growth rates - 12 h - 0,295 h$^{-1}$ for 24 h is 0,364 h$^{-1}$ and the 36$^{th}$ h - 0,237 h$^{-1}$. Strain K32 was isolated from molasses and these results are logical.

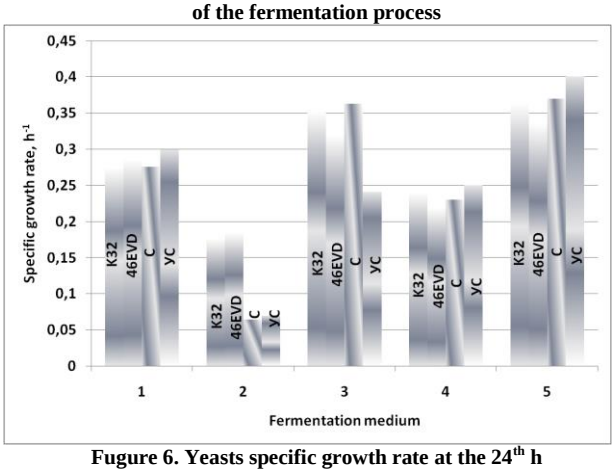
Based on research done in different nutritional environments for strain S. cerevisiae K32 could be made some important conclusions:

- Most suitable for cultivation of S.cerevisiae K32 are nutrient media containing sucrose as a substrate;
- The yeast are longest in the exponential phase of growth in cultivation on molasses. It was observed and maximum growth rate - 0,36 h$^{-1}$.

Similar studies were conducted with *S.cerevisiae* 46EVD. At cultivation in FM 1 the specific growth rate was in the range 0,277-0,298 h$^{-1}$. This rate was achieved even at 12$^{th}$ h of the beginning of the fermentation process and remained relatively high to the 24 $^{th}$ h. However, the rate of accumulation of the product was within the range 1,02 - 1,36 g/(dm$^3$.h), ie accumulation of ethanol occurs intensively.




**Fugure 5. Yeasts specific growth rate at the 12th h of the fermentation process**




**Fugure 6. Yeasts specific growth rate at the 24th h of the fermentation process**

On fermentation medium 2 was observed lower accumulation rate of product in the medium, which remained relatively constant during the active fermentation - within 0,8 g/(dm$^3$.h). In this medium the maximum specific growth rate was relatively low - 0,17 h$^{-1}$. At the end of the process being monitored reaches 49.5% ethanol yield, relatively high for non-optimized cultivation conditions. In medium 3 was observed a high specific product accumulation rate -1, 53 g/(dm$^3$.h). Under these conditions the strain had specific growth rate - 0,33-0,35 h$^{-1}$. In FM 4 strain accumulates ethanol with high specific rate -1,27 g/(dm$^3$.h), which remained relatively constant throughout the studied period. The maximum specific growth rate, which occurred in that time interval, was 0,241 h$^{-1}$. At cultivation in molasses 46 EVD had shown highest values of specific growth rate of yeast - 0,36 h$^{-1}$ and the rate of fermentation-1,446 g/(dm$^3$.h). From the studies of the dynamics of alcoholic fermentation with S.cerevisiae 46EVD, can make the following major conclusions:

- Investigated strain-producer was developing with a high specific growth rate, on all food tested environments. Overall, 46EVD has a greater potential than K32.
- In cultivation on molasses was observed high values of specific growth rate and rate of fermentation process. In this strain on certain nutrient media yield reaches 50% at 48 h from the beginning of the fermentation process.

Similarly to the previous research has research the dynamics of alcoholic fermentation with strains *S.cerevisiae* C and *S.cerevisiae* YC on initially selected 5 nutrient media.

S.cerevisiae C grows well on medium containing sucrose as a substrate. In these nutrient media was observed product accumulation rate within 1,2-1,55 g/(dm$^3$.h). Maximum rate was observed in cultivation in molasses. In these environments the fermentation rate remains relatively constant in the time interval studies. Yeast growth with specific growth rate in the range 0,24-0,36 h$^{-1}$. The maximum rate was reached between 12 and 24 h from the beginning of the fermentation process. At these conditions at 48 h was reached yields between 32% and 52% from the-theoretical.

S.cerevisiae showed better affinity to glucose as substrate. In these nutrient media the fermentation rate was constant for a long period of time. The values was in the range 0,67-0,95 g/(dm$^3$.h). In these nutrient media strain growth with specific rate in the range 0,29-0,36 h$^{-1}$. Unlike the other yeast strains of *S.cerevisiae* YC does not grow well in molasses. Only 48 h is the specific fermentation rate, which satisfies the requirements for conducting the process. Obviously strain shows good performance only nutrient medium containing glucose as substrate.

4. Conclusions

The work studied the fermentation characteristics of yeast strains S.cerevisiae for alcoholic fermentation for ethanol production for the food and biofuels industries. Form the 9 investigated yeast strains *S.cerevisiae* with prven ethanol synthesis properties, the best qualities of the examined parameters (fermentation rate, ethanol concentration and ethanol yield) showed *S.cerevisiae* K32, *S.cerevisiae* 46 EVD, *S.cerevisiae* YC and *S.cerevisiae* C. By the kinetic characteristics of the strains it was found that, *S.cerevisiae* and K32 S.*cerevisiae* 46 EVD show best performance when using glucose as substrate. All strains grow on a maximum rate of molasses, making them suitable for industrial use in ethanol production. Overall, strains 46 EVD has a greater potential than K32. S.cerevisiae 46 EVD grows well on all nutrient media, while K32 develops better on medium, containing sucrose as substrate. The strain S.cerevisiae C grow well on medium containing sucrose and observed rate of accumulation of product within 1,2-1,55 g/(dm$^3$.h). S.cerevisiae YC showed better affinity for glucose as substrate. The observed rates of fermentation was within the range 0,67 - 0,95 g/(dm$^3$.h). In these nutrient media strain growth with specific growth rate in the range 0,29 - 0,36 h$^{-1}$.

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## REFERENCES

1. Berg C., World fuel ethanol - analysis and outlook, 2004, http://www.distill.com/World-Fuel-Ethanol-A&O-2004.html.
2. Demirbas A., Biofuels sources, biofuel policy, biofuel economy and global biofuel projections, Energy Conversion and Management, 49, 2008, 2106–2116.
3. Directive 2003/30/EC of the European Parliament, May 8, 2003.
4. Kosaric N., F. Vardar-Sukan, The Biotechnology of Ethanol - Classical and Future Applications, Edited by M. Roehr, WILEY-VCH Verlag GmbH, 2001
5. Marinov M. Technology of highly alcoholic beverages and spirit, Academic publisher of University of Food Technologies, Plovdiv, 2005 (in Bulgarian).
6. Murgov I., Z. Denkova. Microbiology, Academic publisher of University of Food Technologies, Plovdiv, 2007 (in Bulgarian).
7. Thomsen A.B., C. Medina, B.K. Ahring., Biotechnology in ethanol production, Risø Energy Report, **2**, 2003.
8. Yarovenko V.L. Tehnology of spirit, Colos-Press, Moscow, 2002 (in Russian).
9. Yarovenko V.L., L.Rovinski, Modelling and optimization of microbiology processes in ethanol production, "Food industry", Moskow, 1978 (in Russian).
10. Zaldivar J.,·J. Nielsen, L. Olsson., Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration Appl Microbiol Biotechnol., 2001, 56, 17–34.
11. www.anton-paar.com